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For :VASCULAR ENDOTHELIAL ZINC FINGER 1 GENE AND PROTEIN AND USES THEREOF

DECLARATION OF DR.HEIDI STUHLMANN

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I, Dr. HEIDI M. STUHLMANN, DECLARE THE FOLLOWING:

1. I am a co-inventor of the above-identified patent application.

2. I am a co-author of the attached manuscript, which describes experiments which (i) investigated the expression of *Vezf1* in the adult vasculature and compared it with that of another endothelial cell marker gene, *flk-1*, and (ii) compared the regulation of these genes in model systems for arterial injury and tumor angiogenesis. Using RNA *in situ* hybridization, *Vezf1* expression in the vasculature was found to be restricted to the endothelium of capillaries and mature vessels. Upon arterial injury, expression of *Vezf1*, but not *flk-1*, was up-regulated in the regenerating endothelium only. During tumor angiogenesis, up-regulation of *Vezf1* was detected throughout the capillary network within tumors induced by human primary carcinoma cell lines grown in nude mice, as well as in metastatic human tumors.

3. Based on the results of experiments described in the attached manuscript, I conclude that *Vezf1* is a marker specific for adult endothelial cells which is expressed normally in

the endothelial cell lining of capillary networks and mature vessels throughout adult tissues. Its expression is upregulated in proliferating endothelial cells during pathological growth, i.e. arterial injury, tumor angiogenesis and atherosclerotic plaque development. Restriction to vascular endothelial cells and upregulation of expression in these cells during normal and pathological proliferation demonstrate that *Vezf1* is a potent marker for angiogenesis. Therefore, *Vezf1* would be useful for identifying endothelial cells and for detecting arterial injury and angiogenesis in histopathologic evaluations. Further, in view of its more uniform expression in endothelial cells from a diversity of tissues and its increased expression in regenerating endothelium , *Vezf1* may be a better marker for endothelial cells than the known endothelial cell marker, *flk-1*.

4. I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that I make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified patent application or any patent issuing thereon.

Date: November 18, 2001

Signed: Heidi Stuhlmann

Dr.Heidi Stuhlmann

Vezf1 is a Novel Marker for Endothelial Cells and is Up-Regulated in Angiogenesis and Arterial Injury

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ABSTRACT

The endothelium of the adult vasculature is normally quiescent, with the exception of the female reproductive cycle. However, in response to appropriate stimuli, the vasculature becomes activated and grows new capillaries by a process called angiogenesis. Adult angiogenesis is involved in processes like wound repair, atherosclerosis, tumor growth and metastases, and arthritis. We identified a novel endothelial gene, *Vezf1*, that encodes a transcription factor with six zinc finger domains [Xiong et al. (1999), Dev. Biol. 206: 123-141]. In this study, we investigated the expression of *Vezf1* in the adult vasculature and compared it with that of another endothelial marker gene, *flk-1*. We further studied their regulation in model systems for arterial injury and tumor angiogenesis. Using RNA *in situ* hybridization, *Vezf1* expression in the vasculature was restricted to the endothelium of the capillaries and mature vessels. Upon arterial injury, expression of *Vezf1*, but not of *flk-1*, was up-regulated in the regenerating endothelium only. During tumor angiogenesis, up-regulation of *Vezf1* was detected throughout the capillary network within tumors induced by human primary carcinoma cell lines grown in nude mice, as well as in metastatic human tumors. We hypothesize that *Vezf1* is a novel endothelial cell marker whose up-regulation may be a critical component of an endothelial cell-specific growth response.

Key Words: Cardiovascular System; Gene Expression Regulation; Vascular Endothelium; Physiologic Angiogenesis; Pathologic Angiogenesis

INTRODUCTION

The adult vasculature consists of a complex network of large and smaller arteries and veins, pre-capillary arterioles and venules, and capillaries. Arteries are lined internally by endothelial cells and are surrounded by a thick basement membrane and a layer of smooth muscle cells that is interspersed with pericytes. In contrast, capillaries consist almost entirely of endothelial cells and are only rarely surrounded by smooth muscle-like pericytes. During embryonic development, the primary vascular plexus is formed principally by two mechanism: vasculogenesis and angiogenesis [for review, see[1,2,3]]. Vasculogenesis involves the *de novo* differentiation of endothelial precursor cells, the angioblasts, and their differentiation into primitive blood vessels. Angiogenesis is the process by which new capillaries sprout from pre-existing vessels. The mature vascular system is further developed by maturation and remodeling of the blood vessels [2,3].

In the adult, the vasculature is normally quiescent, with the exception of physiologic angiogenesis during the female reproductive cycles (ovulation, implantation, pregnancy). Arterial endothelial cells have an extremely low turnover rate (about 1 in every 10^5 cells undergoes cell division[4]). Adult endothelial cells are not post-mitotic and, in response to appropriate stimuli, they can proliferate and form new blood vessels. This angiogenic process occurs under physiologic conditions during the female reproductive cycle and wound healing, as well as under pathologic conditions in solid tumors and metastases, rheumatoid arthritis, retinopathies, hemangiomas, and psoriasis[5,6]. Typically, the sprouting of new capillaries involves activation of quiescent endothelial cells, degradation of basement membrane, proliferation and invasion into the surrounding stroma, migration and differentiation, and inhibition of growth[2,6-8].

Some of the mechanisms underlying vasculogenesis and angiogenesis in the embryo are known to involve signaling factors such as bFGF, VEGF, and angiopoietin 1 and 2, the ephrins, and their respective receptors, and they appear to also regulate angiogenesis in the adult[2,3]. However, much of the regulatory events specific for angiogenesis in the adult, including cell-cycle progression, induction of proteolytic activities, remodeling of cell adhesions and junctions, have not yet been elucidated. Recently, it has been proposed that activation of tumor angiogenesis is controlled by an

"angiogenic switch" mechanism[6,7]. According to the hypothesis, the switch can be turned by a change in the balance of activators (aFGF, bFGF, hypoxia, VEGF, etc.) and inhibitors (angiostatin, endostatin, thrombospondin, etc.). It remains to be determined whether a similar switch controls other processes of adult angiogenesis as well.

We have recently identified a novel murine gene, vascular endothelial zinc finger 1 (*Vezf1*), in a genetic screen for early circulatory system genes in mouse embryonic stem (ES) cells[9,10]. The deduced amino acid sequence contains six zinc finger domains and is homologous to a human transcription factor, DB1[11]. DB1 is believed to be involved in regulating expression of cytokine genes such as interleukin-3. Expression of *Vezf1* during embryonic development is restricted to vascular endothelial cells and their precursors in the blood islands of the visceral yolk sac[9]. Its embryonic expression is largely overlapping with that of the receptor tyrosine kinase *flk-1*[12,13]. Northern blot analysis indicates that *Vezf1* transcripts are also present in adult mice in a wide variety of organs, and in established endothelial and hematopoietic cell lines[9].

In a first attempt to understand the biologic role of *Vezf1* in adults, we undertook a comparative study of the expression of *Vezf1* and *flk-1* in the normal adult vasculature, and in models of arterial injury and tumor angiogenesis. We report here that *Vezf1* expression is specific to the endothelium, and that it is up-regulated in regenerating endothelium of injured vessels and in angiogenic endothelial cells.

MATERIALS AND METHODS

Mice and cell lines

CD-1 mice were obtained from Charles River Laboratories, and nude mice (nu/nu) from the Jackson Laboratories, respectively.

Primary cultures of HUVEC cells were isolated from single umbilical cords as previously described[14]. The cells employed these studies were between passages 2 and 4. HUVECs were maintained in Medium 199 supplemented with 20% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, penicillin (50 IU/ml), streptomycin (50 mg/ml), 50 mg/ml porcine intestinal heparin and crude extract of endothelial cell growth factor (ECGF, 50 μg/ml; Gibco BRL). Human microvascular endothelial cells (HMEC) were obtained from the Center for Disease Control (CDC), Atalanta, GA. HMEC were grown in Dulbecco's modified medium (DMEM), supplemented with 10% FCS, 2 mM L-glutamine, penicillin (50U/ml), streptomycin (50 mg/ml), and ECGF (50 μg/ml). For experiments involving growth factor induction, HUVEC were incubated in a defined medium consisting of DME with bovine serum albumin (BSA) (25 μg/mL), ascorbate (0.2 mM), insulin (1 μM) and transferrin (5 μg/mL)[15]. All the growth factors were purchased from R&D. The breast cancer line MDA-MB-435, a highly metastatic subclone from line MDA-MB-231 and the prostate cancer line DU145 were obtained from the ATCC. Tumor cell lines were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml).

Generation of tumors in nude mice

Nude mice (10 mice for each tumor cell line) were inoculated subcutaneously in the region of the mammary fat pad with $5x10^6$ MDA-MB-231, or DU145 cells, respectively. Tumors were propagated for 6-8 weeks, until they reached about 5 to 12 cm in diameter. Animals were sacrificed, and tumors and normal tissue were subjected to histopathological analysis. Samples were fixed overnight in 3% formalin, embedded in paraffin at 60°C following a series of dehydrating washes in ethanol and a final 2x 45 min wash in xylene. Material was sectioned at 4 μm.

Human tumor and carotid artery biopsies

Human tissues were obtained from excess surgical pathology specimens (atherectomies, endarterectomies from two individuals) and autopsy specimens (metastatic breast tumors from two indiviuals diagnosed with ductal carcinomas) at the Mount Sinai Medical Center. Formalin-fixed, paraffin-embedded tissue blocks were serially sectioned at 5 µm onto lysine-coated slides and stored at room temperature until used. Before staining, the slides were heated at 57°C for 2 hrs, rinsed in xylene for 10 min, rehydrated in successive rinses of graded ethanols, and placed in phosphate-buffered saline (PBS). Samples were prepared for *in situ* hybridization as described above for the tumors grown in nude mice.

Balloon injury

Aortic balloon injury was performed in adult male Sprague-Dawley rats (300-350 gm) under general anesthesia (intraperitoneal ketamine 15 mg/kg) as previously described[16]. A #2 Fogarty balloon embolectomy catheter was introduced via the femoral artery and advanced to the level of the aortic arch. It was then inflated and withdrawn along the full length of the thoracoabdominal aorta. This procedure was repeated five times. Following single injury, the femoral artery was ligated, the incision closed, and the animals returned to their usual diet and routine. Untreated control rats, and at 48 hrs, 1 week, 2 weeks, 4 weeks, and 6 weeks injury, respectively, rats were anesthetized and heparinized, and the ascending aorta were cannulated and perfused with 200 ml heparinized PBS followed by 200 ml of 4% paraformaldehyde in PBS, pH 7.4.

Preparation of adult mouse organs and rat arteries for RNA *in situ* hybridization and immunostaining

CD-1 adult mouse organs and rat arteries were harvested on ice, washed in PBS, and fixed overnight in 4% paraformaldehyde. Samples were embedded in paraffin at 60°C following a series of dehydrating washes in ethanol and a final 2x 45 min wash in xylene. Material was sectioned at 4 µm.

Plasmid DNA and riboprobes

Sense and antisense riboprobes for mouse *Vezf1*, corresponding to nucleotide 1397 to 2899, have been described[9]. A probe specific for the human homologue of *Vezf1*, *DB1* or *VEZF1*, was generated by subcloning an EcoRI fragment (nucleotides 1051-2306) from EST AA 195025 (obtained from Genome Systems) into pBluescript (Stratagene). Sense and antisense riboprobes were synthesized from plasmid DNA that was linearized with NotI or EcoRI, and transcribed from the T7 or T3 promoter, respectively. A probe specific for *flk-1* has been described previously[13].

RNA *in situ* hybridization

Procedures for RNA *in situ* hybridization were essentially as described previously[10,17]. Briefly, sectioned material was de-paraffinized and digested with proteinase K, followed by a 16 hr. hybridization with ^{35}S-radiolabeled antisense riboprobes (final probe concentration was 35 dpm/ml in hybridization buffer). Post-hybridization washes of increasing stringency were included to reduce background. Sense control probes were synthesized for all experiments. Slides were dipped in Kodak NBT-2 emulsion, dried over-night and exposed for 7 days at 4°C. Material was counter-stained with toludine blue, or hematoxylin and eosin, respectively. Dark field photographs were taken using a Leica Leitz DMRB Microscope. Bright Field photographs were taken using a Zeiss Axioskop Microscope.

RNA preparation and Northern blot hybridization

Extraction of total RNA, agarose gel electrophoresis, transfer to nitrocellulose membranes and hybridization to [a-32P]dCTP-labeled DNA were as previously described[18]. Prehybridization and hybridization were performed at 42°C. Hybridization was performed with a 770 bp fragment of human *VEZF1* (corresponding to nucleotides 3 to 776 of the DB1 cDNA sequence[11]), and a mouse GAPDH cDNA plasmid, respectively, that were labeled by random hexamer priming to a specific activity of >10^8 cpm/mg and used at 2 x 10^6 cpm/ml. Final washes were in 1x SSC (0.15M NaCl/15 mM sodium citrate, pH 7.0), 0.1% SDS at room temperature for 1 h.

Antibodies and immunohistochemistry

Polyclonal antibodies against the N-terminal region of mouse VEZF1 were raised in rabbits (Cocalico Biologicals, Inc.). Briefly, a PCR-amplified fragment corresponding to amino acids 1-169 was subcloned into pET 28b, and recombinant protein was induced and purified on divalent Ni^{2+} agarose beads. A purified 27kD band, corresponding to the recombinant protein, was used for inoculation of rabbits. Polyclonal antibodies from the rabbit immune serum were further purified by immunoaffinity purification using bacterially expressed VEZF1 1-169 peptide. Sections from rat arteries and nude mouse tumors were processed as described (Schechter et al. 2000) and immunostained for VEZF1 (1:10 dil.) and ICAM (1:50 dil.). Nonimmune negative and control slides without primary antibodies were prepared for each antigen stain. Slides were counterstained with hematoxylin, coverslipped, and examined.

RESULTS

Differential levels of *Vezf1* expression in the quiescent adult endothelium

In a previous study using Northern analysis of total RNA of adult mice, we showed that substantial levels of *Vezf1* transcripts were present in all adult organs tested[9]. To investigate the spatial distribution of *Vezf1* transcripts in adult organs, we performed RNA *in situ* hybridization on sections of adult mouse organs.

Vezf1 expression was detected in quiescent endothelium of every adult mouse tissue examined. Expression was primarily confined to the endothelial lining of the capillary network (Fig. 1) and was less prominent within the endothelium of the larger, more mature blood vessels (not shown). Compared to its high level of expression during embryogenesis[9], the levels of *Vezf1* transcripts detected by *in situ* hybridization in interstitial capillaries of adult organs was considerably lower. Sections of adult organs that were hybridized to a *flk-1* riboprobe did not detect any signals above background level (not shown). *Vezf1* signal was present in the capillaries infusing the cardiomyocytes (Fig. 1A) as well as within the visceral pericardium and endocardium of the heart (not shown). Likewise, *Vezf1* expression was detected in the endothelial lining of skeletal muscle (Fig. 1B). The highest level of *Vezf1* expression in adult mice was found in the lung in capillaries surrounding the alveolar septae (Fig. 1C,G,H); this correlated with the high abundance of *Vezf1*-specific mRNA in the lung seen on Northern blots[9]. Expression of *Vezf1* in the kidney and liver (Fig. 1D,E) and in the spleen (not shown) was found in interstitial spaces between large cells, presumably localized to the flat endothelium of the capillary network. In contrast, no *Vezf1* signal was detected in hepatocytes, macrophages or hematopoetic cells of the liver or spleen (Fig. 1E). In the brain, and in the reproductive tract (uterus, oviduct and ovaries) of a 10 week-old mouse, *Vezf1* transcripts were restricted to the capillary spaces (not shown). In placenta at day-16.5 of gestation, abundant *Vezf1* expression was found within the capillary network of both maternal and fetal origin (not shown). Low levels of *Vezf1* signal were detected in the bone marrow within the megakaryocytes (Fig. 1F), whereas expression in other hematopoietic cells was at background levels. No *Vezf1* expression was detected in peripheral blood.

Up-regulation of *Vezf1* during tumor angiogenesis in a nude mouse model system

A human breast cancer cell line (MDA-MB435), and a human prostate cancer cell line (DU145) were used to induce tumor formation in nude mice. RNA *in situ* hybridization using a mouse *Vezf1*-specific riboprobe was performed on sections from two tumors derived from the breast cancer line (tumors #8549 and 8550) and one tumor from the prostate cancer line (tumor #8451C). *Vezf1* expression was markedly elevated within the capillaries infusing the growing tumors derived from the breast cancer cell line when compared to its expression in the surrounding parenchyme (Fig. 2A,B). High levels of *Vezf1* expression were detected within the capillary spaces at the periphery (Fig. 2A,B) and in the interior of the tumors (not shown), but not in the tumor cells. A human-specific *VEZF1* riboprobe did not detect any signal above background levels when tested on sections of E10.5 mouse embryos, as well as in the tumor cells or infusing capillaries of the nude mouse tumors (not shown). In contrast to *Vezf1*, very low levels of *flk-1* expression were found in the breast tumor and adjacent normal tissue (Fig. 2C,D). Similarly, sections through the tumor derived from the prostate cancer cell line showed elevated *Vezf1* expression within the tumor vascular network (Fig. 2E,F), whereas *flk-1* expression was low (not shown).

High levels of *VEZF1* expression in human tumor biopsies

A human-specific riboprobe was used to examine *VEZF1* expression in two metastatic human breast tumors. *VEZF1* signal was detected at low levels in the surrounding normal adventitia, and was dramatically increased within the interstitial endothelial network between tumor cells (Fig. 3A,B,C). In contrast, biopsies derived from primary tumors of the lung, prostate, and from a glioblastoma did not reveal any discernible increase in *VEZF1* mRNA levels within the tumor tissue (not shown).

Up-regulation of *Vezf1* in response to arterial injury

Next we examined the expression of *Vezf1* and *flk-1* mRNA in regenerating endothelium during response to balloon injury of rat aortas. In normal arteries, *Vezf1* signal was distributed uniformly in the endothelium, and was also detected in small patches surrounding adventitial capillaries (compare Figure 4A to 4C). No expression was found in the medial layer of smooth muscle cells (SMC). In contrast to *Vezf1*, no

signal was seen in the endothelium of control arteries using a *flk-1*-specific riboprobe (Figure 4D). 48 hrs after denudation of the endothelium, *Vezf1* transcripts were not detected on the luminal surface or in the medial SMC (Fig. 4E). Two weeks after injury, there was substantial intimal hyperplasia and almost complete endothelial regeneration. This was accompanied by intense *Vezf1* expression localized in the endothelium, but not in the SMC of the developing intima or the media (Fig. 4F,G). A decrease in *Vezf1* expression in the regenerating endothelium was seen at 4 and 6 weeks after injury (Fig. 4I). No *flk-1*-specific transcripts were detected at 2 or 4 weeks after injury in the regenerating endothelium or SMC (Fig. 4H, J).

To localize the VEZF1 protein within cells of the injured arteries, we used polyclonal antibodies raised against the N-terminal region of VEZF1 for immunohistochemistry. Low levels of protein expression were detected in the luminal endothelial layer of control arteries (Fig. 6A). Strong VEZF1 expression was found in regenerating endothelial cells, but not in the medial SMC or in the devloping intima, 2 weeks and 4 weeks after injury (Fig.6B,C). Interestingly, the protein was localized both, in the nucleus and cytoplasm of endothelial cells. In contrast, ICAM expression was detected in the luminal endothelium as well as the underlying cell layer of newly developing intima in arteries 4 weeks post-injury (Fig. 6D).

Vezf1 expression in human carotid artery plaques

VEZF1 expression was also examined in human atherosclerotic plaques. Substantial levels of *VEZF1* expression were detected in the luminal endothelium, but not in the underlying SMC (Fig. 5A,B). Immuno-histochemical staining of parallel sections using anti-human smooth muscle-specific α-actin antibodies, or endothelial-specific anti-vWF antibodies, respectively, confirmed restricted localization of *Vezf1* mRNA to the endothelium (not shown).

VEZF1 expression in human umbillical vein endothelial cells (HUVEC)

Several growth factors, including tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), and vascular endothelial growth factor (VEGF), have been implicated in regulating endothelial growth during tumor angiogenesis and endothelial regeneration after arterial injury. In addition, these factors are early activators of gene

expression in endothelial cell culture. To determine if these agents were early activators of *VEZF1* expression, 80% confluent cultures of HUVEC were treated in defined medium with TNFα (100 ng/ µl), TGFβ (100 ng/µl), or VEGF (50 ng/ µl). As exemplified in Figure 7, these agents did not induce or repress *VEZF1* mRNA expression in cultured HMEC cells over a 12 hr time period. In addition, there were no changes in the levels of *Vezf1* mRNA at 3, 6, 12, 24, 36, or 48 hrs after HUVEC were incubated in 10% FBS (data not shown). No differences in the response to growth factors or serum were seen whether the cells were treated 24 hrs after incubation in fresh growth medium (containing 10% FBS) or in medium containing 1% FBS. Similar results were found using cultures of HMEC.

DISCUSSION

Angiogenesis, the formation of new capillaries and vessels by sprouting or splitting from preexisting vessels, is essential for the growth of normal and neoplastic tissues. Angiogenesis involves proteolytic degradation of the extracellular matrix, chemotactic migration and proliferation of endothelial cells, and formation of a new lumen and maturation of the endothelium (reviewed in[2,19,5,7]). Several key players in both embryonic and adult angiogenesis have been identified as growth factor ligands and their tyrosine kinase receptors participating in signaling pathways, including VEGF[20] and its receptors *flk-1*[21,22] and *flt-1*[23], and angiopoietin 1 [24,25] and 2[26] and its receptor *tie2*[27],[28]. Although the importance of these signaling pathways for both physiologic and pathologic angiogenesis has been recognized, many questions remain unanswered. For example, their levels of expression do not always correlate with endothelial proliferation. Therefore, other factors that function either independent of, or in the same signaling pathways as VEGF, Ang 1 and 2, and their receptors, might be involved in regulating endothelial proliferation.

The murine zinc finger gene *Vezf1* was identified in our genetic screen for early circulatory system genes in ES cells and mouse embryos[29]. Its restricted expression during embryogenesis in the vascular endothelium and its precursors in the yolk sac blood islands suggested that *Vezf1* plays a role during endothelial lineage determination, and possibly during embryonic vasculogenesis and angiogenesis[9]. In previous reports, using Northern blot analysis of total RNA, transcripts specific for *Vezf1* and its human homologue, DB1, were found in all adult organs tested, and in several established endothelial and hematopoietic cell lines[9,11]. In this report, we show that *Vezf1* expression is maintained throughout the quiescent endothelium of the adult vasculature, and that expression is up-regulated during vascular injury and endothelial proliferation during tumor angiogenesis.

Using RNA *in situ* hybridization, *Vezf1* mRNA was detected at similar levels in all adult tissues examined, with the highest expression in the lung. *Vezf1* expression appeared to be restricted to the endothelium of the quiescent vasculature, with expression highest in capillaries and lowest in mature vessels. In comparison to embryonic vessels, overall expression of *Vezf1* is reduced in the adult endothelium. No

expression was detected in the intimal smooth muscle cells. Expression of *flk-1* during embryogenesis is similar, but not identical to that of *Vezf1*. Whereas *Vezf1* expression is restricted to the putative mesodermal hemangioblasts in the yolk sac membrane and the endothelium [[9], S. Robertson, G. Keller, and H. Stuhlmann, unpublished], *flk-1* is also expressed transiently in primitive hematopoietic cells and the endocardial tubes[21,13,30,31]. Furthermore, expression of *flk-1* varies and becomes down-regulated to low or undetectable levels in the endothelium of many adult organs, including brain, lung, thymus, bone marrow, uterus, and testes[21,12,32,33,34]. Due to its more uniform tissue expression, *Vezf1* may be a better marker for the endothelium than *flk-1*.

A second site of low level *Vezf1* expression was detected in a small subpopulation of cells in the bone marrow and thymus, in agreement with previously reported expression of *Vezf1*/DB1 in various established hematopoietic and lymphatic cell lines[9,11]. The close apposition of hematopoietic and endothelial cells in the developing vascular system and their presumed coomon precursor, the hemangioblast, might be reflected in shared transcriptional regulatory mechanisms. In contrast, *Vezf1* expression was not detectable in primitive hematopoietic cells of the fetal liver, or in peripheral blood cells during embryogenesis[9] or adults. Thus, these results may indicate a secondary role for *Vezf1* during adult hematopoiesis.

Little is known about the biologic role of *Vezf1* during development or in the adult endothelium. Its human homologue, DB1, was identified as a DNA binding activity to a GC-rich region in the IL-3 promoter and is believed to be involved in regulating the activity of cytokine genes[11]. In support of the notion that *Vezf1* encodes a 56 KD putative zinc finger transcription factor, we have identified a strong transactivation domain in a proline-rich region at the C-terminus of VEZF (X. Sun, J.-W. Xiong, and H. Stuhlmann, manuscript in preparation). However, no target genes have been identified that are regulated *in vivo* by *Vezf1* or DB1. It is possible that *Vezf1* acts in the VEGF signaling pathway, either upstream or downstream of its receptor tyrosine kinases. Because *Vezf1* and *flk-1* are differently regulated in adult angiogenesis, and expression of *Vezf1* in endothelial cells does not respond to growth factor and cytokines that regulate *flk-1* expression, it is likely that they participate in different regulatory pathways. Identification of factors that regulate *Vezf1* expression, and of target genes for VEZF may help elucidate its biologic function.

Up-regulation of *Vezf1* expression was detected during arterial injury and tumor angiogenesis. It remains to be determined whether *Vezf1* up-regulation occurs in physiologic angiogenesis associated with the ovarian cycle and pregnancy as well. In arterial injury and tumor angiogenesis, expression remained restricted to the endothelium and was not detectable in intimal SMC, or tumor cells, respectively. Thus, *Vezf1* is a marker specific for adult endothelial cells within the arterial wall. VEZF protein might be a potential target to modulate endothelial cell proliferation in conditions of normal and pathologic angiogenesis, independent of the intimal smooth muscle cells. In addition, regulatory elements within the *Vezf1* promoter might be useful tools for treating vascular disorders by targeting gene expression specifically to the endothelial cells.

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FIGURE LEGENDS

Figure 1. Expression of *Vezf1* in organ sections of adult CD-1 mice.

A mouse-specific, antisense *Vezf1* riboprobe labeled with [^{35}S] UTP indicates levels of gene expression in the endothelium of: heart myocardium (A); skeletal muscle (B); lung (C, G, H); kidney (D); liver (E); bone marrow (F). Sections were counter- stained with hematoxylin and eosin, and photographed under bright field at 63X (A, C, D, E, F, and G) or 20X (B), respectively. (G) and (H) depict bright field and dark field images, respectively, of sections from lung, photographed at 40X. Arrows in (A, B) depict the position of *Vezf1* signal in the interstitial space between syncitial myocytes.

Figure 2. Expression of *Vezf1* and *flk-1* in breast and prostate tumor cell lines grown in nude mice.

A mouse *Vezf1* antisense riboprobe labeled with [^{35}S] UTP indicates levels of gene expression in capillaries of the periphery of tumor #8549 derived from breast cancer line MDA-MB435 (A), and of tumor #8451C derived from prostate cancer line DU145 (E). A mouse *flk-1*-specific antisense riboprobe labeled with [^{35}S] UTP indicates levels of gene expression in the periphery of tumor #8549 derived from breast cancer line MDA-MB435 (C). 63X bright field photomicrographs from *in situ* hybridization (A,C,E) in the left panel were stained with toludine blue and correspond to nearby sections immunostained for mouse ICAM and counter-stained with hematoxylin (B,D,F), in the right panel. Photomicrographs A and C were taken from adjacent sections of tumor #8549.

Figure 3. Expression of *Vezf1* in human breast tumor biopsies.

A human [^{35}S-UTP] labeled *VEZF1* antisense probe was hybridized to sections from a metastatic breast tumor. Post hybridization, samples were stained with hematoxylin and eosin. Photomicrographs were taken at 40X (A) under dark field, or at 40X (B) and 63X (C) under bright field optics, respectively.

T, tumor tissue; N, normal tissue surrounding the tumor.

Figure 4. Expression of *Vezf1* and *flk-1* in untreated control and balloon-injured rat arterial sections.

Untreated control rat artery stained with hematoxylin and eosin (A). Control sense *Vezf1* riboprobe labeled with [^{35}S-UTP] on untreated control rat artery (B). Mouse-specific antisense *Vezf1* riboprobe labeled with [^{35}S-UTP] indicate levels of gene expression in an untreated control rat artery (C) and the balloon-injured rat arteries at: 48 hours post-injury (E); 2 weeks post-injury (F,G); and 4 weeks post-injury (I). Antisense *flk-1* riboprobes labeled with [^{35}S-UTP] indicate gene expression in control rat arteries (D); 2 weeks post-injury (H); and 4 weeks post- injury (J). 10X photomicrographs A, bright field and B, C, D, E, F, I, and J, dark field. 40X bright field photomicrographs G and H, stained with toludine blue.

Figure 5. Expression of *VEZF1* in human arterial plaques.

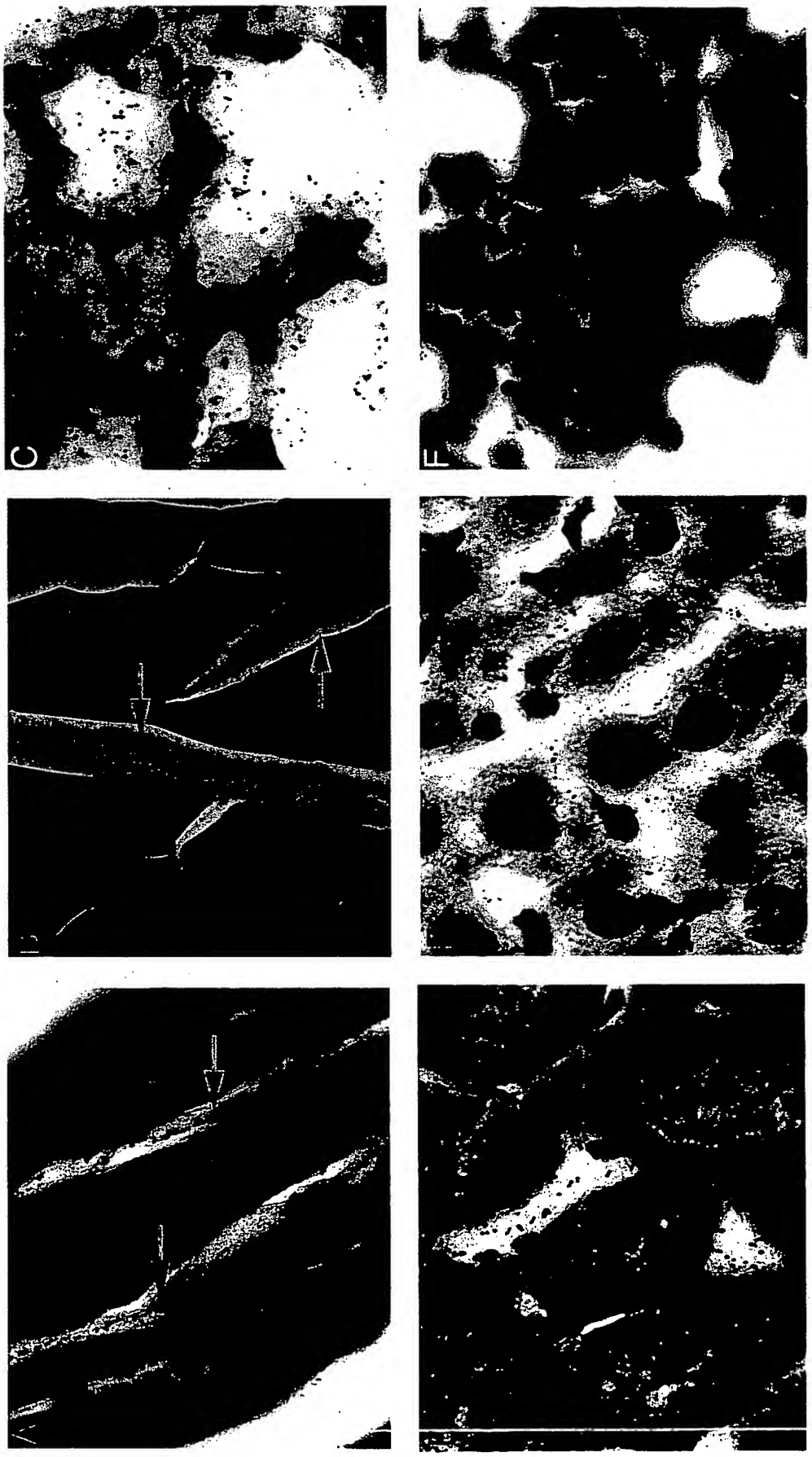
Human-specific antisense *VEZF1* riboprobe labeled with [^{35}S-UTP] indicates levels of gene expression in carotid artery samples from patient : 7079 (A) and 2799 (B).

Figure 6. Localization of VEZF1 protein in untreated control and balloon-injured rat arterial sections.

Sections with untreated control rat artery (A), and arteries 2 weeks (B) and 4 weeks (C) post-injury were immunostained for VEZF1 using immunoaffinity-purified, polyclonal antibodies against VEZF1 (amino acids 1-169) and counterstained with hematoxylin). (D) A section from a rat artery 4 weeks post-injury adjacent to that shown in (C) was immunostained for ICAM and hematoxylin-counterstained.

Figure 7. *VEZF1* mRNA expression in HUVEC cells.

HUVEC (80% confluent cultures) were treated in defined medium with TNFα (100ng/ml), TGFβ (100 ng/ml), or VEGF (50 ng/ml). Total RNA was isolated between 1 to 12 hrs later from cells grown in parallel cultures. Northern blots were probed with a [α-^{32}P]-labeled fragment specific for human *VEZF1*, and a labeled GAPDH cDNA plasmid.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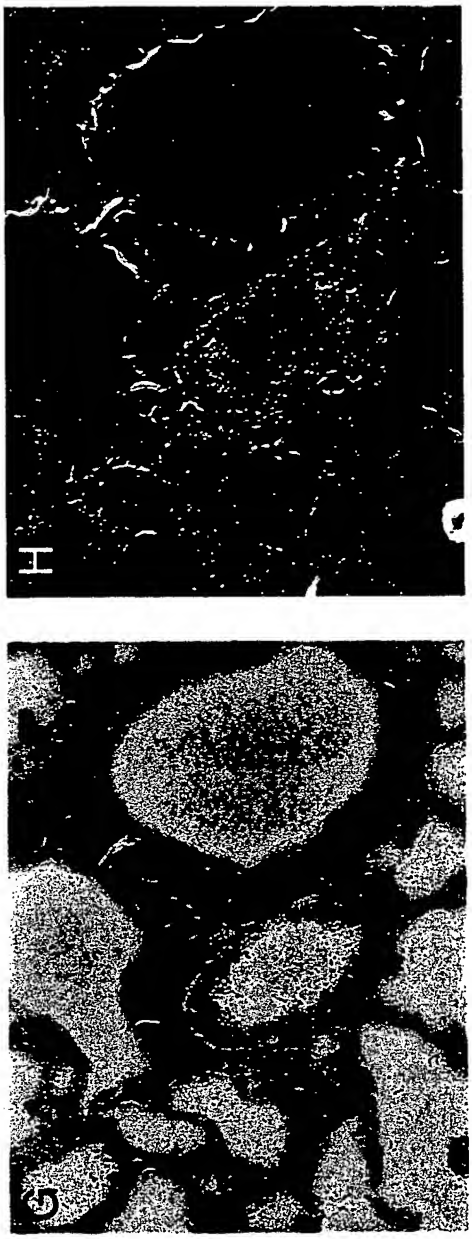
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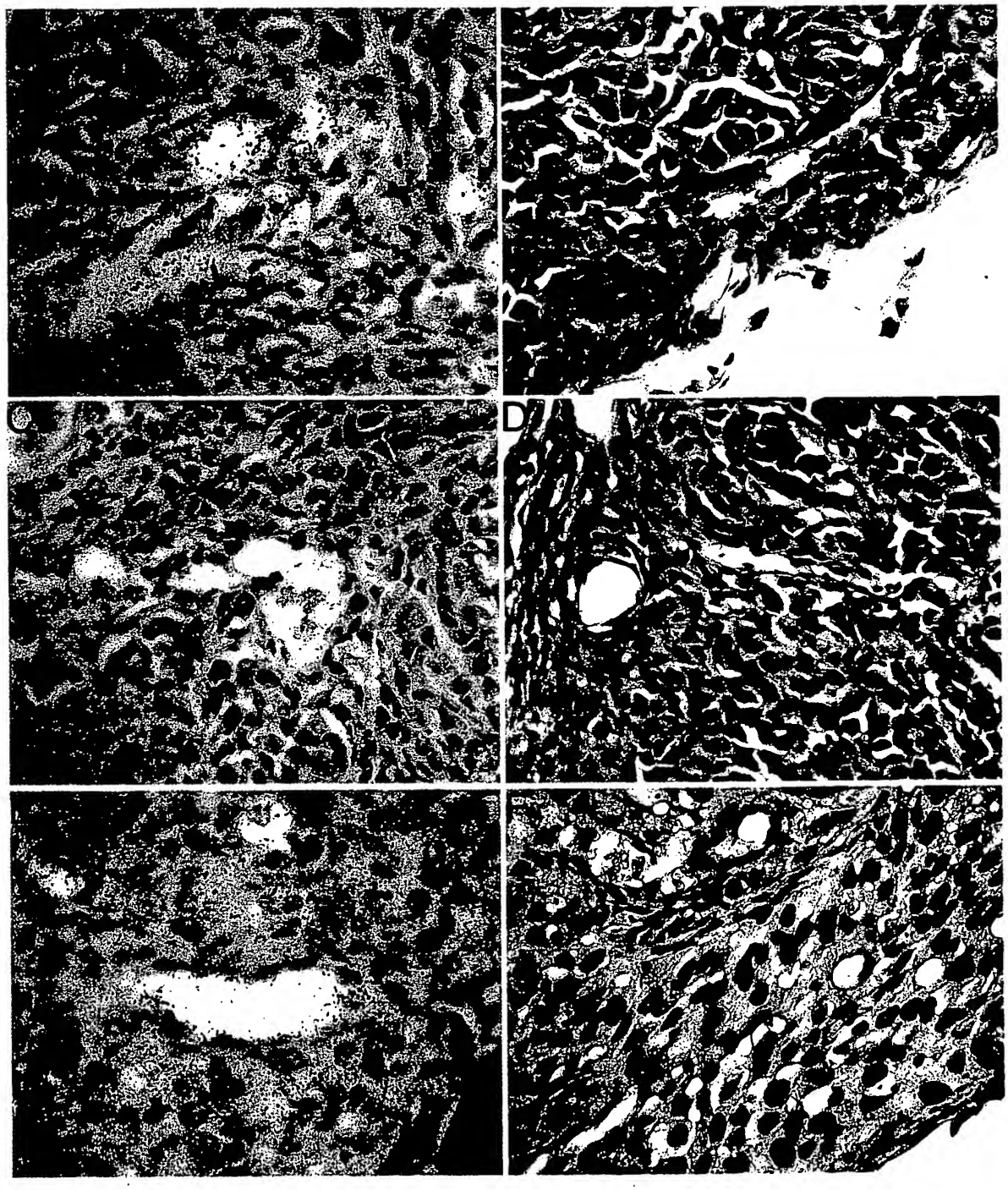
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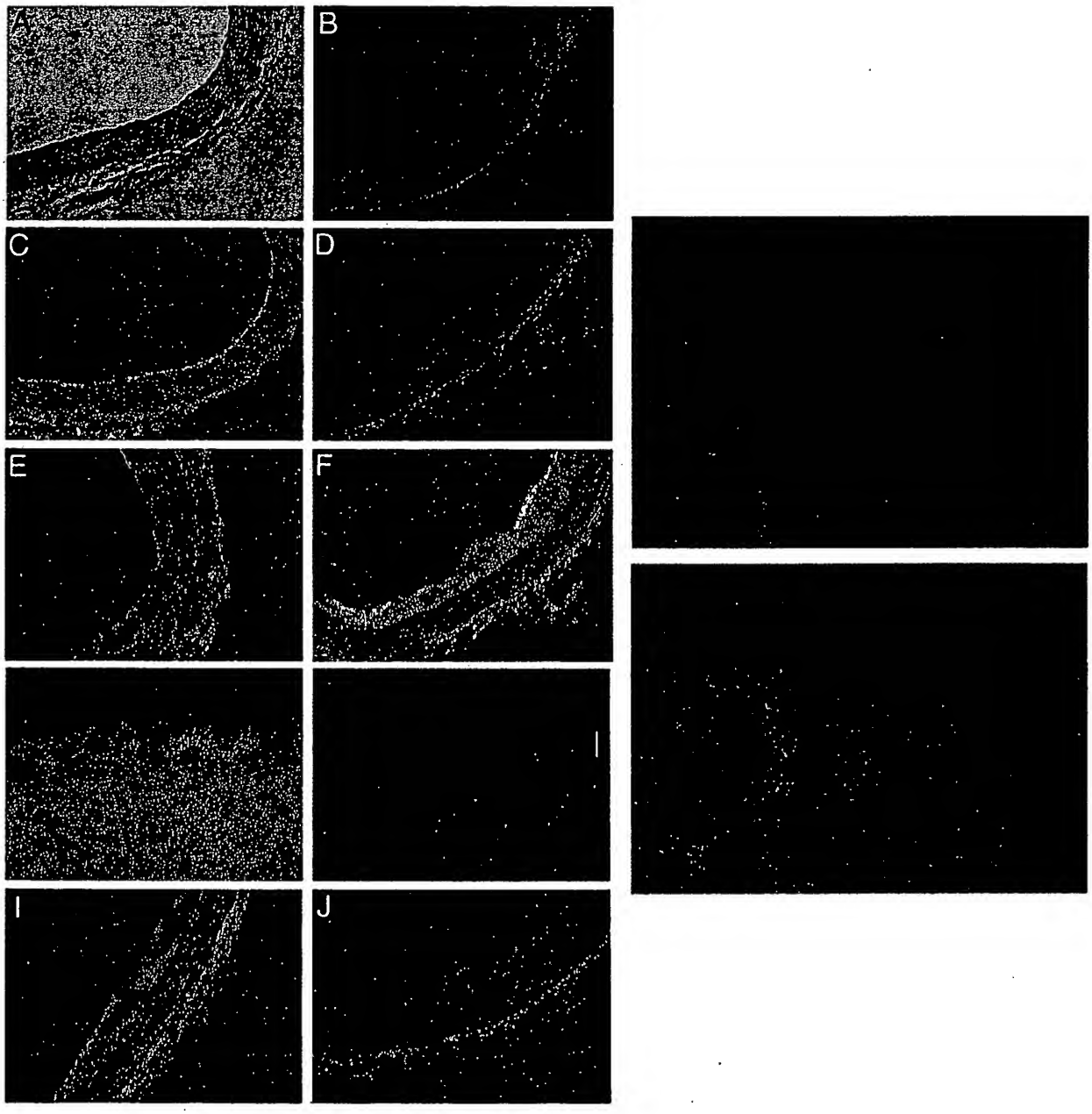
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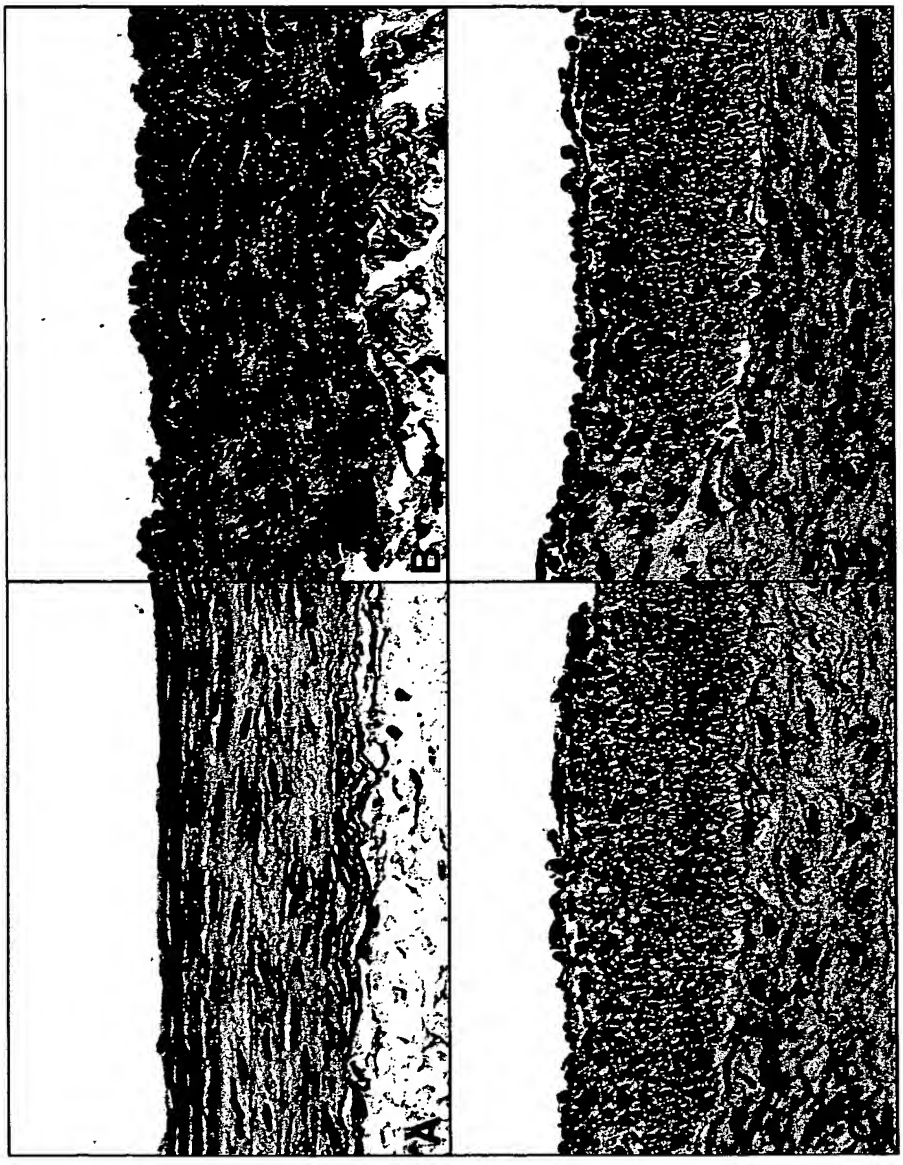
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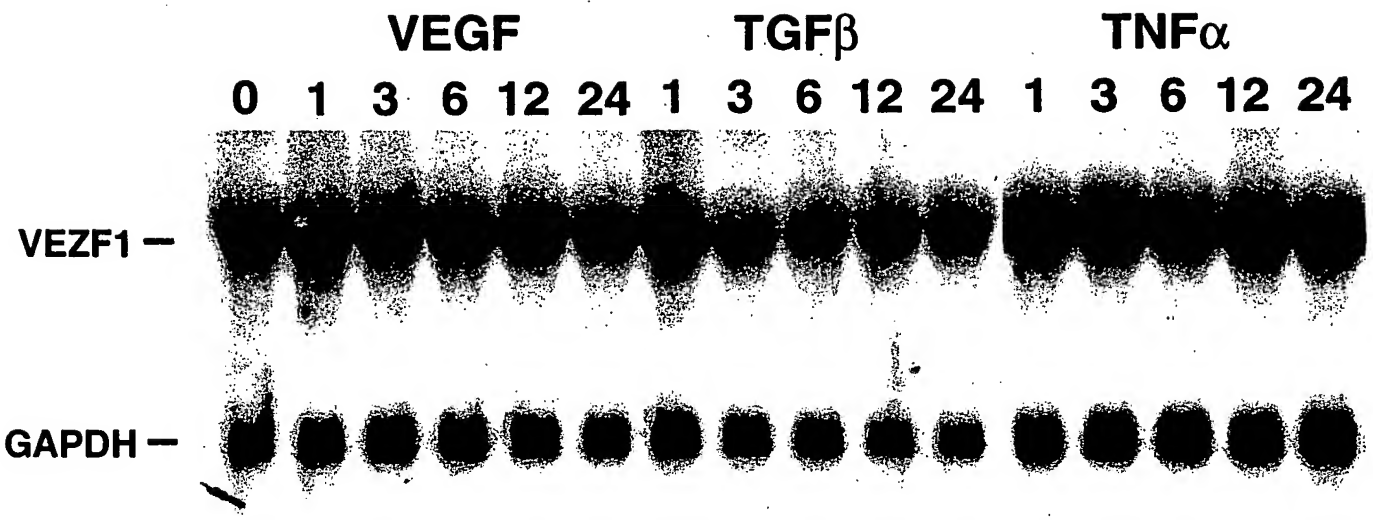
VEGF
TGFβ
TNFα
0 1 3 6 12 24 1 3 6 12 24 1 3 6 12 24
VEZF1
GAPDH